Original Article

Expression and Purification of Functionally Active Recombinant Human Alpha 1-Antitrypsin in Methylotrophic Yeast *Pichia pastoris*

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Abstract

Human alpha 1-antitrypsin (AAT) cDNA was obtained from HepG2 cell lines. After PCR and construction of expression vector pPICZα-AAT, human AAT was expressed in the yeast *Pichia pastoris* (*P.pastoris*) in a secretary manner and under the control of inducible alcohol oxidase 1 (*AOX1*) promoter. The amount of AAT protein in medium was measured as 60 *mg/l* 72 *hr* after induction with methanol. Results indicated the presence of protease inhibitory function of the protein against elastase. Purification was done using His-tag affinity chromatography. Due to the different patterns of glycosylation in yeast and human, the recombinant AAT showed different SDS-PAGE patterns compared to that of serum-derived AAT while pI shifted from 4.9 in native AAT compared to 5.2 in recombinant AAT constructed in this study.

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Introduction

Human alpha 1-antitrypsin (AAT) is the major member of serin protease inhibitor (serpin) superfamily in plasma which is mainly produced in liver [(1)]. It is now recognized that AAT is a potent inhibitor of multiple serine proteases, and protects tissues against their harmful effects. The average concentration of AAT in plasma is 1.3 *mg/ml*, with a half-life of 3 to 5 days. Individuals with plasma AAT values below 0.7 *mg/ml* are considered to be AAT deficient [(2,3)]. Intravenous administration of a pasteurized pooled human plasma AAT product (Prolastin; Bayer Corporation) is used to increase AAT levels in deficient individuals [(4)]. This approach of therapy is practical and feasible [(5,6)]. However, there are two major obstacles; namely source limitation and risk for emerging viruses [(7)]. Alternatively, the recombinant versions of AAT have been under intensive investigation.

Since the early 1980s, the human AAT gene has been expressed in various hosts, including *Escherichia coli* (*E.coli*), yeasts, insect cells, CHO cells, as well as in transgenic plants and animals. Protein size, glycosylation pattern, metastable inhibitory nature of AAT and production cost represent the challenges in the production of recombinant AAT [(8-12)].

We considered the metylotrophic yeast *P.pastoris* as an attractive host for production of human AAT. It is a single-cell eukaryote with the advantages of both prokaryotic and

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eukaryotic hosts. One of its main advantages is the ability for introducing many of post-translational modifications such as glycosylation and proteolytic processing which is obtained through moving in the secretory pathway [13]. For glycoproteins such as AAT, these modifications are very important for appropriate function and/or structure [14,15].

In the present study, the AAT was expressed in *P.pastoris* as a fusion protein to a histidine tag (His-tag) which facilitates purification step. Signal sequence from *Saccharomyces cerevisiae* α-mating factor (α-MF) was included to direct secretion of the protein to extracellular medium. This powerful expression system made use of the highly inducible alcohol oxidase 1 (*AOX1*) promoter to express large quantities of glycosylated protein. The *P.pastoris* produced AAT activity and its features were evaluated for the first time using elastase inhibitory assay and Isoelectric Focusing (IEF), respectively. Mobility shift caused by addition of tunicamycin-a N-linked glycosylation inhibitor- to culture indicated the effect of glycosylation on the protein weight and SDS-PAGE protein band pattern. These data will be used in future studies for generating a new recombinant AAT protein with pharmaceutical objectives.

Materials and Methods

Bacteria and P.pastoris strains and media

The *E.coli* strain DH5α was used for propagation of recombinant plasmids. The *P.pastoris* strain X-33 (Invitrogen) was used as a host for the protein expression. Recombinant bacteria were cultured in low salt LB broth medium (0.5% (*w/v*) yeast extract, 1% (*w/v*) tryptone, 0.5% (*w/v*) NaCl) supplemented with 25 $\mu g\ ml^{-1}$ of zeocin (Invitrogen). *P.pastoris* was cultured on the following media: YPDS plates (1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 2% (*w/v*) dextrose, 1 *M* sorbitol, and 1.5% (*w/v*) bacteriological agar) supplemented with 100 $\mu g\ ml^{-1}$ of zeocin; Buffered Glycerol-complex Medium (BMGY) medium (1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 100 *mM* potassium phosphate, 1.34% (*w/v*) Yeast Nitrogen Base (YNB), 4×10^{-5}% (*w/v*) biotin, 1% (*v/v*) glycerol, pH=6.0); Buffered Methanol-complex Medium (BMMY) medium (1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 100 *mM* potassium phosphate, 1.34% (*w/v*) YNB, 4×10^{-5}% (*w/v*) biotin, 0.5% (*v/v*) methanol, pH=6.0) supplemented with 2% Phenyl Methyl Sulphonyl Fluoride (PMSF); Minimal Dextrose (MD) agar (1.34% (*w/v*) YNB, 4×10^{-5}% (*w/v*) biotin, 2% (*w/v*) dextrose, 2% (*w/v*) agar); and Minimal Methanol (MM) agar (1.34% (*w/v*) YNB, 4×10^{-5}% (*w/v*) biotin, 0.5% (*v/v*) methanol, 2% (*w/v*) agar).

Cloning of AAT in pPICZαB plasmid

Since the hepatic cells are the main source for AAT expression, total RNA was extracted from human hepatocellular carcinoma (Hep G2) cell line (obtained from the Pasteur Institute of Iran, Tehran). Briefly, about 10^7 cells were harvested and total RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instruction. Primers 1 (5′CTC TCGAGAAAAGAGAGGCTGAAGCTGAA GATCCCCAGGGAGATG 3′) and 2 (5′GC TACTCTAGATAATTTTTGGGTGGGATT CAC 3′) were employed to amplify AAT cDNA excluding its signal peptide (amino acid residues 1-24) from the 100 ng RNA in 20 μl reaction using the One-Step RT-PCR Kit (Qiagen). In designing the primers, restriction sites of *Xho*I and *Xba*I were incorporated at the 5′ ends of the PCR products. The cDNA product was cloned into the pPICZαB plasmid (Invitrogen) in a way to introduce the His-tag epitope at the carboxyl terminal of the recombinant AAT.

P.pastoris transformation and selection

P.pastoris cells were grown overnight in YPD broth (at 30 °*C*/ 250 *rpm*) and prepared for transformation according to the manufacturer's recommendations (Invitrogen). The recombinant plasmid was linearized with *Sac*I and electrotransformed into the 80 μl of competent *P.pastoris* using a Bio-Rad gene-pulser apparatus (at 1.5 *kV*, 25 *F*, 400 Ω and 8 *ms*). Hundreds of transformed cells were

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selected by growing on YPD agar plate in presence of 100 $\mu g\ ml^{-1}$ of zeocin.

Determination of methanol utilizing (Mut) phenotype

Transformation of *P.pastoris* X-33 cells with linearized DNA results in two phenoltypes of host cells in respect with methanol utilization (Mut). Since single crossover recombination at the *AOX1* promoter region is favored, most of the transformants have Mut^+ phenotype in which the *AOX1* gene is intact. However, due to the presence of the *AOX1* terminator sequences in the plasmid, there is a chance for second recombination at this region which result in replacing of *AOX1* gene with desired one hence creating Mut^S phenotype. The latter is weak in metabolizing methanol. The Mut phenotype for the transformed X-33 cells was determined by growing one hundred clones on minimal media with methanol (MM) or dextrose (MD) plates. Chromosomal integration of the plasmid DNA and right orientation of the expression cassette containing the AAT cDNA were characterized by PCR analysis and DNA sequencing. 5′ and 3′ *AOX1* primers (5' GAC TGGTTCCAATTGACAAGC 3' and 5' GC AAATGGCATTCTGACATCC 3'), directed to the *AOX1* promoter and the transcription terminator were used for this purpose.

Shake flask protein expression

Ten verified transformants were grown at 30°*C* in 10 *ml* BMGY medium for 24 *hr* until reaching an OD_{600} of 10. The cells were harvested and resuspended in 50 *ml* BMMY medium containing 0.5% (*v/v*) methanol. After incubating the cells at 30*°C*/200 *rpm* for 72 *hr* methanol was added at 1% (*v/v*) once a day to maintain induction.

SDS-PAGE and silver staining

The cells were removed and the supernatant was precipitated by using 100% Trichloroacetic Acid (TCA) solution. After drying, the pellet was resuspended in loading buffer containing β-mercaptoethanol, heated for 10 *min*, in boiling water and electrophoresed at 12% SDS-PAGE/100 *V* along with molecular weight marker (Fermentas). Gel was stained with silver nitrate according to Celis and coworkers [16].

Western blot analysis

Protein samples resolved on SDS-PAGE, were electro-blotted to Polyvinylidene Difluoride (PVDF) membrane (Millipore) in transferring buffer (0.025 *M* Tris, 0.19 *M* glycine, and 20% (*v/v*) methanol) overnight at 20 *V*/4°*C*. The membrane was treated with PBS-T-BSA (PBS, 0.1% (*v/v*) Tween 20, 1% (*w/v*) BSA) for 2 *hr* to block binding sites. After washing step, membrane was reacted with 1000-fold diluted goat anti-human alpha-1 antitrypsin polyclonal antibody**,** conjugated with HRP (Abcam) for 3 *hr*. To eliminate non specific reactions, a supernatant of non- recombinant X-33 culture treated side by side accordingly as negative control. Subsequently, protein bands reacted positively, were visualized at the presence of 4-chloro1-naphtol substrate in PBS.

Quantification of AAT

The Enzyme-linked Immunosorbent Assay (ELISA) was used for quantitative determination of secreted AAT levels in the medium using human alpha 1-antitrypsin ELISA Quantitation Kit (GenWay Biotech, Inc) according to the manufacturer's recommendations. For all experiments the samples were diluted 200 folds in sample/conjugate diluents (50 *mM* Tris, 0.14 *M* NaCl, 1% (*w/v*) BSA, 0.05% (*v/v*) Tween 20, pH=8.0). Different concentrations of commercial human AAT (Sigma) were used to construct standard curve, while supernatant of non-recombinant *P.pastoris* (X-33 strain) culture was used as a negative control.

Affinity chromatography purification

For the purification of His-tag fused AAT, the supernatant was applied to a nickel-immobilized chelating sepharose fast flow column (Amersham, Biosciences). For this purpose supernatant first was diluted with equal volume of 2X binding buffer (50 *mM* NaH_2PO_4, 500 *mM* NaCl, 10 *mM* imidazole, pH=7.4) and loaded on to the column. After passing

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the wash buffer (50 *mM* NaH_2PO_4, 500 *mM* NaCl, a gradient of imidazole from 20 to 40 *mM*, and 0.05% (*v/v*) Tween 20, pH=7.4) through the column, the resin-bounded recombinant AAT was eluted with elution buffer (50 *mM* NaH_2PO_4, 500 *mM* NaCl, 250 *mM* imidazole, and 0.05% (*v/v*) Tween 20, pH=7.4).

Inhibitory activity assay

Elastase activity was measured by EnzChek® Elastase Assay Kit (Molecular Probes, Inc.) according to the manufacturer's recommendations. The EnzChek kit contains DQ™ elastin soluble bovine neck ligament elastin that has been labeled with BODIPY® FL dye such that the conjugate's fluorescence is quenched. The non-fluorescent substrate can be digested by elastase or other proteases to yield highly fluorescent fragments. The presence of an inhibitor such as AAT blocks the substrate digestion hence subsequent fluorescent emission. The resulting change in fluorescence level was monitored using a standard fluorometer (Hitachi F-3010) with a maximum absorption at 505 *nm* and a maximum fluorescence emission at 515 *nm*. Commercial human AAT was used as a positive control and the elution buffer and the supernatant from non-recombinant *P.pastoris* (X-33 strain) culture as negative controls.

Isoelectrofocusing (IEF)

IEF was accomplished on a pharmacia flatbed electrophoresis apparatus at 4°*C* using high voltage and carrier pharmalytes (Sigma) over the pH range of 4.5-5.4. The experiment was performed on the 7% polyacrylamide gradient gel. Subsequently, the IEF gel was stained with Coomassie brilliant blue R-250.

Inhibiting protein glycosylation using tunicamycin

Tunicamycin was used to block *in vivo* glycosylation. To determine differences in glycosylation of the recombinant protein, tunicamycin (Sigma, T7765) was used at final concentration of 2.5 *μg/ml* in BMMY culture medium. Cell density was checked every day and after 96 *hr* of induction with methanol, the supernatant was precipitated using 100% TCA solution. The results of SDS–PAGE and western blotting were compared to those of *P.pastoris* at the same conditions except no tunicamycin.

Results

AAT cloning in P.pastoris

The cDNA of AAT from HepG2 cell line was cloned in frame with the *α*-MF secretion signal into the expression vector pPICz*α*B. The resulting plasmid was named pPICZα-AAT. Transformants were selected on YPDS agar containing zeocin, and the Mut phenoltype was determined by growing them on selective MD and MM media. According to corresponding data, more than 90% of transformants had equal growth features on both media that is a proof for Mut^+ phenotype. PCR results with *AOX1* primers for genomic DNA were consistent with those of screening in selective media and confirmed that the AAT gene had been integrated into the *AOX1* locus. The correct orientation and nucleotide sequence of AAT cDNA within the *P.pastoris* genome verified by sequencing. Mut^+ clones showed two different bands in agarose gel electrophoresis, the 1.8 *kb* fragment which was amplified from AAT expression cassette flanked by *AOX1* sequences and another 2.2 *kb* fragment which was amplified from *AOX1* gene of *P.pastoris*. In Mut^S clones only 1.8 *kb* band was observed, which demonstrates the disruption of *AOX1* gene (Figure 1).

SDS-PAGE and AAT purification

Supernatant samples from cultures of few positive clones and one negative clone (non-recombinant X-33) were subjected to SDS-PAGE analysis after precipitation with TCA. The results provided an additional band near 60 *kDa* only in positive clones (Figure 2, Lanes 1-6). The secreted protein was purified from the supernatant by passing through a nickel column and fractions were screened by SDS-PAGE. The purified protein samples were seen as two or three smeared bands after Coomassie brilliant blue R-250 staining (Figure 3A).

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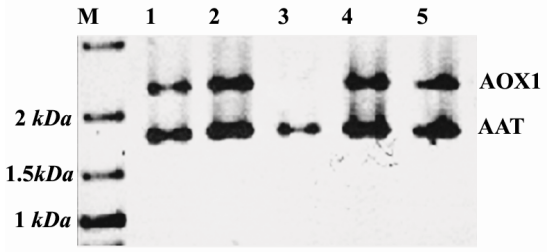

Figure 1. PCR with *AOX1* primers from recombinant *P.pastoris* genome. Lanes 1, 2, 4 and 5 belong to Mut$^+$ clones showing two different bands in the gel. The 1.8 *kb* band was amplified from AAT expression cassette flanked by *AOX1* sequences, and the 2.2 *kb* band was amplified from *AOX1* gene of *P.pastoris*. Lane 3 belongs to a Mut^S clone where only the 1.8 *kb* band is seen. This confirms that *AOX1* gene has been disrupted in these cells

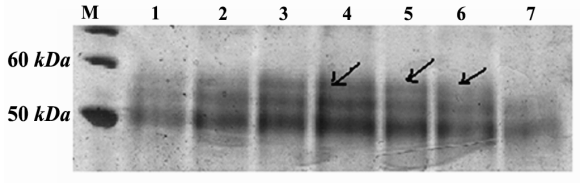

Figure 2. SDS-PAGE and silver staining of supernatants of positive clones. M) protein molecular weight marker; Lanes 1-6: supernatants of positive clones which show an extra band in ~60=*kDa*; Lane 7: supernatant of the non-recombinant X-33 clone, used as negative control

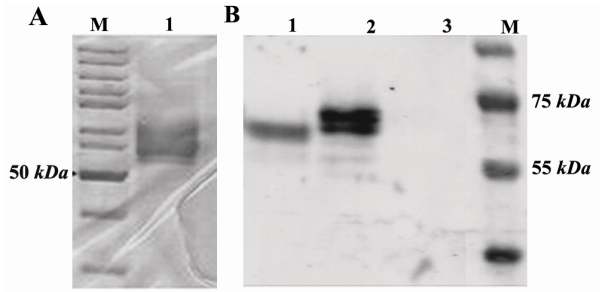

Figure 3. A) SDS-PAGE analysis of protein purification with nickel column stained with Coomassie brilliant blue. M: protein ladder; Lane1: nickel column-purified protein. B) Western blotting of AAT protein on PVDF membrane. M: protein ladder; Lane 1: commercial plasma derived AAT; Lane 2: nickel column-purified recombinant AAT protein; Lane 3: supernatant of non-recombinant X-33 culture

Immunoblot assays

To confirm the identity of recombinant protein, the purified protein was reacted with AAT antibody in a western blot analysis. The recombinant AAT was identified as two distinct bands (Figure 3B, Lane 2) with a molecular weight slightly larger than that of the commercial protein (Figure 3B, Lane 1).

AAT levels in supernatant were determined by quantitative ELISA, using two specific monoclonal antibodies against AAT. Level of protein expression was determined in ten Mut$^+$, three Mut^S, and one non-recombinant X-33 clones. According to our data, methanol induction at concentration of 0.5% (*v/v*) for the first day and 1% (*v/v*) for the next two days could result in highest level of protein secretion in Mut$^+$ phenotype. Average quantities of protein expression were 60 *mg/l* and 44 *mg/l* after 72 *hr* for Mut$^+$ and Mut^S clones, respectively (Figure 4).

Functional assay and IEF

Data from the activity assays provided an increase in detected fluorescence levels when the elution buffer and/or the supernatant of non-recombinant X-33 culture were screened. However, no changes were seen for commercial AAT and the recombinant AAT (Figure 5).

After staining with Coomassie brilliant dye, recombinant AAT protein was visualized as three bands in IEF acrylamide gel migrating at 5.1 to 5.2 *kDa*, while plasma derived AAT was detected as five bands from 4.7 to 4.9 *kDa* (data was not shown).

Effect of tunicamycin

To investigate whether the protein bands with higher molecular weight were due to dif-

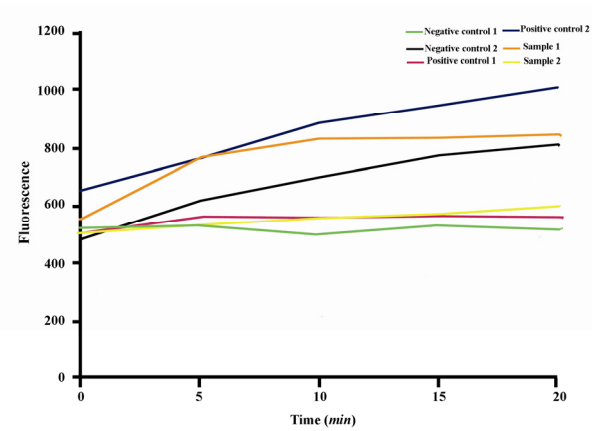

Figure 4. Comparison of quantities for secreted recombinant AAT in Mut$^+$, Mut^S and non-recombinant X-33 clones where identified by human alpha 1-antitrypsin ELISA Quantitation Kit in three consecutive days. Data represent the average of three independent experiments

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ferent glycosylation, we expressed the AAT in media containing tunicamycin which blocks glycosylation *in vivo*, and analysed the supernatant by SDS-PAGE and western blotting. In the presence of tunicamycin the AAT band shifted to a position lowers than that of natural and recombinant proteins (Figure 6).

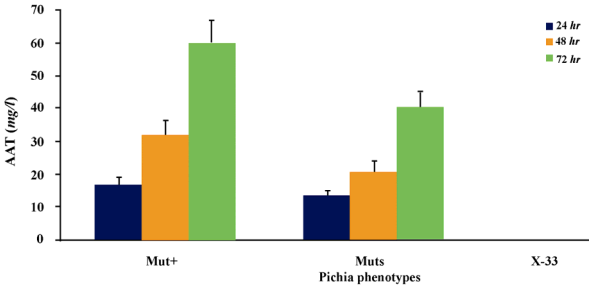

Figure 5. Activity assay of the recombinant AAT Commercial plasma-derived AAT were used in 10 and 20 μg concentrations as positive controls 1 and 2; supernatant of non-recombinant X-33 and elution buffer were used as negative controls 1 and 2, respectively; 20 μg of the recombinant AAT from two different clones were used for inhibitory activity analysis. Active AAT inhibited increasing of fluorescence level

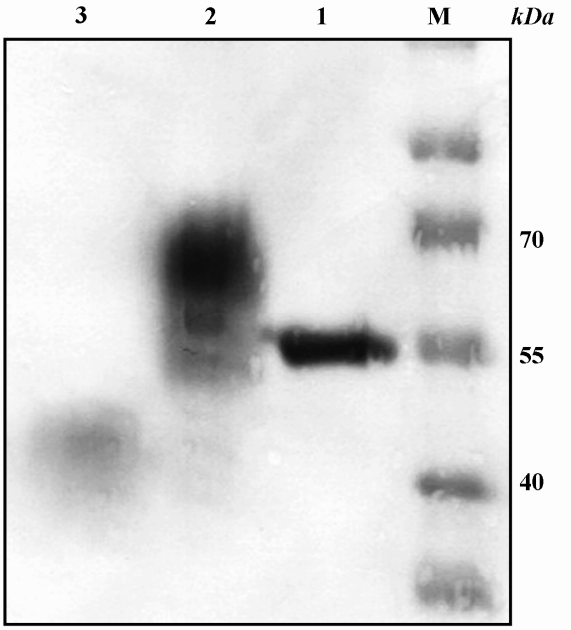

Figure 6. Expression of AAT protein in the absence and presence of tunicamycin monitored by western blot analysis. M: protein ladder; Lane 1: commercial plasma-derived AAT; Lane 2: control recombinant AAT protein; Lane 3: recombinant AAT produced in culture containing 2.5 $\mu g/ml$ tunicamycin. Weakness of this band is due to inhibitory effects of tunicamycin for yeast growth with subsequently lowers cell density and secreted proteins in the medium

Discussion

The methylotrophic yeast *P.pastoris*, has emerged as an important and efficient host in modern biotechnology. It has been used as a successful expression system for recombinant protein in academic research and industrial production [17,18]. A large variety of proteins that cannot be expressed in *E.coli* at the correct level of post-translational maturation have been subsequently produced in the eukaryotic cells [19,20]. The glycosylation pattern in *P.pastoris* is different from that of mammalians and this may or may not be a problem depending on the target protein and its application.

AAT is a glycoprotein and addition of carbohydrate moieties to appropriate positions is an important post-translational modification that is essential for the stability of AAT protein [21,22]. Therefore, to obtain proper glycosylation is one of the major concerns in the production of recombinant AAT. The most suitable candidates for production of AAT in correct form with closest modification to authentic human AAT are mammalian cells [7]. But using these cells for production of recombinant protein is a time consuming process and its industrial/ pharmaceutical application is not cost efficient. Using yeasts like *P.pastoris* for production of AAT glycoprotein seems to be an effective alternative and has resulted higher yields. *P.pastoris* in comparison with two other yeasts (*Saccharomyces cerevisiae* and *Hansenula polymorpha*) produces recombinant AAT with the glycosylation pattern closer to that of the authentic human AAT [23]. Furthermore, *P.pastoris* core oligosaccharides were reported to have no immunogenic terminal α-1,3 glycan linkages [24]. We selected metylotrophic yeast *P.pastoris* for production of AAT and according to our data this system could potentially be used as an effective host for this purpose.

The AAT was produced in quantities of up to 60 *mg/l* in shake flask. In SDS-PAGE, our recombinant protein showed a different migration pattern in comparison to that of plasma AAT. This could be the result of dif-

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ferent patterns of glycosylation. Tunicamycin treatment which inhibits the glycosylation of secretary proteins, confirmed this idea. These differences in glycosylation pattern affected the mobility of recombinant protein on IEF and increased its pI in comparison to plasma derived AAT from 4.2-4.9 [25,26] to 5.1-5.2.

Conclusion

Activity assay demonstrated the protease inhibitory power for this recombinant protein. The high mannose glycosylation apparently did not alter the main function of the protein. Besides, no change on AAT secretion was observed. The immunogenic properties of this protein will be studied for its medicinal potential. Further studies are needed to screen protein expression while increasing the production scale.

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